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Simultaneous enhancement of thermostability and catalytic activity of phospholipase A_1 by evolutionary molecular engineering

Jae Kwang Song and Joon Shick Rhee

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1, Kusong-dong, Yusong-gu, Taejon 305-701, Korea

Abstract

The thermal stability and catalytic activity of phospholipase A_1 from *Serratia* sp. MK1 were improved by an evolutionary molecular engineering. Two thermostable mutants were isolated after sequential rounds of error-prone PCR to introduce random mutations and filter-based screening of the resultant mutant library, and identified as having six (mutant TA3) and seven (mutant TA13) amino acid substitutions, respectively. Different types of the substitutions were found in two mutants, resulting in the increase of nonploar residues (mutant TA3) or changes between side chains within polar or charged residues (mutant TA13). The wild-type and mutant enzymes were purified, and the effect of temperature on their stability and catalytic activity was investigated. The T_m values of TA3 and TA13 were increased by 7 and 11°C, respectively. Thus, evolutionary molecular engineering was found to be an effective and efficient approach to increasing thermostability without compromising enzyme activity.

Introduction

Enzymes can be tailored for optimal performance in industrial applications by evolutionary molecular engineering, also called directed evolution or *in vitro* evolution (Arnold and Moore, 1997; Rubingh, 1997). Many successfully engineered proteins have been reported, and especially, enzymes with enhanced properties such as sufficient stability (Giver *et al.*, 1998; Zhao and Arnold, 1999), high activity (Taguchi *et al.*, 1998), altered substrate specificity (Yano *et al.*, 1998) and the ability to interact correctly with surfaces (Egmond *et al.*, 1996) have been developed for industrial applications (Arnold and Volkov, 1999). Judging from the recent successes in irrational design approaches such as mutagenic PCR (Cadwell and Joyce, 1992) and DNA shuffling (Stemmer, 1994) followed by screening for improved properties, directed evolution may be more efficient than rational design involving both iterative computer design and site-directed mutagenesis.

Especially, thermostability is often a primary goal in improving the properties of an industrial enzyme, since high temperatures in industrial processes impart such benefits as increased substrate solubility, decreased viscosity of the medium or lower risk of microbial contamination. In other words, thermostable enzymes are of considerable biotechnological interest as their enhanced stability could greatly reduce enzyme replacement costs or permit processes to be carried out at high temperatures. Recently, many attempts have been made to understand the principles of the stability of proteins: the introduction of disulfide bonds (Reiter *et al.*, 1995), chemical cross-links (Noda *et al.*, 1997), salt bridges (Fairman *et al.*, 1996) and metal binding sites (Pantoliano *et al.*, 1988) and the increase of intramolecular hydrophobic packing (Yutani *et al.*, 1987) have been proposed. However, the application of these principles is limited to the enzymes whose 3-D structures have been determined. Especially in the case of enzymes without the extensive information on structure and function, directed evolution is a powerful tool for studying or engineering thermostability and catalytic activity of the enzymes.

Phospholipase A_1 hydrolyzes 1-acyl group of a phospholipid to lysophospholipid and fatty acid. In recent years, it has been reported that phospholipase A_1 as well as well-characterized phospholipase A_2 plays an important biological role in both phospholipidosis, a pathological condition in which phospholipids accumulate in lysosomes (Reasor and Kacew, 1996), and virulence factors for bacterial and fungal pathogenesis (Hoffman DR, 1994). In addition to its physiological roles, phospholipase A_1 is of particular interest in industrial application as it yields 2-acyl-lysophospholipids. Lysophospholipids as excellent emulsifiers are particularly suitable for use in many industrial applications, such as in food technology, or in the cosmetics and pharmaceutical industries. Lysophospholipids show the ability to enhance emulsification properties in oil/water emulsions due to an increased solubility in water, form emulsions which are more stable to changing pH and temperature conditions, and maintain emulsions stable in the presence of magnesium or calcium ions. Lysophospholipids also have several physiological functions, such as its role in platelet aggregation and its role as a signaling molecule (Durieux and Lynch, 1993). They also affect ripening and storage characteristics of fruit, leaves and green plant tissue (Palta and Farag, 1992).

The phospholipase A_1 gene, *plaA* from *Serratia* sp. MK1 encodes a monomer of 321 amino acids and has been cloned as described in our previous study (Song *et al.*, 1999). Even though the presence of phospholipase A_1 has been reported in various sources (Brok *et al.*, 1994; Dawson *et al.*, 1983; Hoffman DR, 1994; Pete *et al.*, 1994; Schmiel *et al.*, 1998), the

application of phospholipase A_1 in phospholipid modification has been limited due to its low stability and poor availability. Moreover, crystallographic and structural data of phospholipase A_1 have not been elucidated as yet. Since evolutionary protein engineering technique was an alternative and more useful approach for modifying enzymes in the absence of such knowledge, we, therefore, attempted to use evolutionary molecular engineering to improve phospholipase A_1 for practical purposes.

Evolutionary engineering used in this study was the technique of preparing protein variants by using mutagenic PCR, expressing the protein and then screening for those with improved thermostability. In the present study, we enhanced catalytic activity as well as thermostability of phospholipase A_1 from *Serratia* sp. MK1 by the evolutionary technique. For this purpose, we developed a filter-based screening system that could identify both properties on single processed filter, in which catalytic activity of heat-treated mutant library was assayed on the phosphatidylcholine-containing gel at normal temperature. We reported two thermostable phospholipase A_1 mutants which exhibit higher activity at temperature range examined than that of the wild-type (Song and Rhee, 2000). Details are presented in this paper (Song and Rhee, 2000).

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